Table 1.

Claim Element (by reference to Claim 16)	**Component used in present tests**	**Citation examples in specification**
Bacterial cell harboring the whole set of the *tra* transfer genes on its chromosome	*E. coli* strain S17-1	*E. coli* as donor cell: page 13, lines 25-17.
Transmissible plasmid comprising	pCON4-47, pCON4-44	Embodied in the Description of the Invention, as a whole,
An origin of replication	RK2 *oriV*	*oriV* teachings at Page 10, lines 8-21, including RK2 specifically
An origin of transfer	RK2 *oriT*	*oriT* teachings at Page 9, lines 2-3; attached Fig. 2
A screenable marker gene	TetR, AmpR, KanR,(antibiotic resistance markers);	Page 13, line 15 (general teaching of antibiotic resistance genes as suitable screenable markers)
One or more transfer genes conferring the on the donor bacterium the ability to conjugatively transfer the transmissible plasmid to the recipient bacterium	Tra1 and Tra2 on plasmid pCON4-47	Sources for transfer (*tra*) genes: Page 11, lines 3-14
"Killer gene"	*colE3*	"Killer genes", generally at page 11, line 15- page 13, line 6; *colE3*, known in the art at the time of filing: (Bowman et al. Proc Natl Acad Sci 68:964-968, 1971)
Pathogenic recipient bacterium	*E. coli* 0157:H7	*E. coli* linked to enteritis, page 15, lines 25-26
Pathogenic recipient bacterium	*Salmonella typhimurium*	*Salmonella typhimurium* at page 15, line 26

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Marcin S. Filutowicz	Group Art Unit:	1645
Serial No.:	09/651,290	Examiner:	Vanessa L. Ford
Filed:	August 30, 2000	File No.:	960296.00084

For: ANTI-MICROBIAL BIOTHERAPEUTIC AGENTS: ALTERNATIVES TO CONVENTIONAL PHARMACEUTICAL ANTIBIOTICS

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner For Patents
Alexandria, VA 22313-1450

Dear Sir:

I, Hideki Suzuki, on oath say and declare that:

1. I am currently employed by ConjuGon, Inc. (Madison, WI) as its director for the microbiology department. ConjuGon, Inc. is a startup biotechnology company specializing in anti-infectives and is the licensee of the technology described in the present patent application.

2. I obtained my Ph.D degree in microbiology in 1988 from University of Tokyo, Japan, and had my postdoctoral training at Ohio State University and Cornell University. I have worked as a research scientist specializing in the general area of biotechnology for 16 years. For the last three and a half years, my research has focused on anti-infectives. I have published extensively in the area of microbiology and is familiar with state of the technology at the time the application was filed.

3. I have reviewed the Office Action issued in this matter by the U.S. Patent and Trademark Office on March 19, 2004. I understand that claims 1-12 and 16-27 are rejected for the alleged lack of enablement, in part because the Examiner believes that the specification does not provide enough evidence to demonstrate that a skilled artisan would be able to practice the invention in the "real world" by following the teachings of the

specification. This declaration along with the experimental data attached are submitted to provide evidence that the specification enabled the "real world" use as specified in the application.

4. To demonstrate this, I as well as other employees of ConjuGon who are under my supervision conducted experiments for several "real world" uses as specified in the application. We simply followed the teachings of application and successfully killed two strains of pathogenic bacteria on flower petals, leaves, meat surfaces, sliced potatoes, and in sheep blood plasma and human urines.

5. At the time the application was filed, the art of microbiology had developed to a stage such that recombinant plasmids and bacterial cells had been routinely constructed for various purposes including for controlling plasmid replication, for transferring a plasmid from a donor cell to a recipient cell, for selecting desirable recombination events, for controlling gene expression, and so on.

6. The donor and recipient cells used in the experiments are those described in the specification. The transmissible plasmids were also constructed based on the teachings of the specification. The exact parts of the specification that teach the donor and recipient cells as well as the plasmids that were employed in the experiments are detailed below. A summary is provided in Table 1. Certain experimental details to the extent that they are not described in the specification were all well known and routine to a skilled artisan at the time the application was filed. Therefore their inclusion in the specification was not necessary for enablement. This point is further supported below with the noted references that disclosed the relevant techniques prior to the filing of the application.

7. For the experimentation attached, killing was through the use of "killer genes" as described at page 11, line 15 to page 13, line 6, and also as described in claim 16. A non-self-transmissible plasmid, pCON4-44, and a self-transmissible plasmid, pCON4-47, were used for the experiments. The genetic elements of these plasmids are shown in attached Fig. 2. As taught on page 9 at lines 12-19 of the application, non-self transmissible plasmids generally comprise an *oriT*, an *oriV* and one or more screenable markers. The non-self transmissible plasmid used in these tests, pCON4-44, comprises *oriT*, and an *oriV* and includes the *TetR* tetracycline resistance gene as a screenable marker. As taught on page 9 at lines 20-27, self-transmissible plasmids generally comprise these same elements, and additionally comprise *tra* transfer genes. The self-transmissible plasmid used in these tests, pCON4-47, comprises *oriT*, *oriV* and the TetR screenable marker, and further comprises Tra1 and Tra2 regions encoding the whole set of the *tra* transfer genes.

8. *colE3* was used as a "killer gene". Colicin E3 is a ribonuclease that cleaves 16S ribosomal RNA, inhibiting synthesis of proteins in the cell and leading to cell death, which were well known in the art at the time the application was filed (Diaz et al. Mol. Microbiol 13:855-861, 1994). This *colE3* gene was integrated in the two above plasmids (pCON4-44 and pCON4-47) using standard cloning techniques, in accordance with the methods of Sambrook and Ausubel, as cited at page 16, lines 23-27 of the present application. A self-transmissible control plasmid, pCON4-45, was also constructed. pCON4-45 does not carry the "killer gene" but confers tetracycline resistance via conjugation, allowing conjugation efficiencies to be monitored without killing the recipient cell.

9. Three donor strains, each carrying one of the plasmids described above, were tested for conjugative transfer and recipient cell killing ability in and on a variety of biological samples and tissues including flower petals, leaves, meat surfaces, sliced potatoes, plasma, and urines. The donor strains were genetically engineered to contain abundant repressor to suppress the expression of the "killer gene" (*colE3*) based on the teaching in the specification (page 4, lines 14 and 14, page 13, lines 7-9, and claims 16 and 19). Abundance of repressor was well known in the art at the time of filing (Muller-Hill et al. Proc Natl Acad Sci 59:1259-1264, 1968; Amann et al. Gene 69:301-315, 1988; Kleiner et al. J Gen Microbiol 134:1779-84, 1988).. The donor cells were also modified to contain the toxin-immunity protein ImmE3 (which can bind to ColE3 to prevent its toxic effect) so that any leaky expression of *colE3* would not cause any detrimental effect to the donor cells. Use of ImmE3 to prevent toxic effects of ColE3 was well known in the art when the application was filed (Bowman et al. Proc Natl Acad Sci 68:964-968, 1971; Masaki and Ohta. J Mol Biol 182:217-227, 1985).

10. In these experiments, two pathogenic bacteria (*Escherichia. coli* O157:H7 and *Salmonella typhimurium*, also referred to as *Salmonella enterica* serotype Typhimurium) were used as targets. These target pathogens are disclosed at page 15, lines 25-26.

11. In these experiments, equal amounts of donor cells carrying one of the "killer plasmids" (pCON4-44 or pCON4-47) and the pathogenic bacterial cells were mixed and spun down to a pellet. The pellet of mixed bacteria was re-suspended in plasma or urine. For testing on the surfaces of flower petals, leaves, meat, and sliced potatoes, the mixed bacterial pellet was re-suspended in a small volume of saline and spread on the surfaces. After one hour of incubated at 37°C, the mixture of bacteria was serially diluted, and survival of the target bacteria was monitored by growing them on nutrient-rich agar plates containing

appropriate antibiotics. Unlike the filter conjugation described by Merryweather, which was standard and well known in the art at the time the application was filed (J. Bacteriol. Merryweather et al., 167:12-17, 1986; J. Bacteriol. Haase et al,. 177:4779-4791, 1995), these experiments were carried out within or on the surface of biological materials. An illustration of the method to monitor conjugation-dependent killing is shown in Fig. 1. The results of conjugation-dependent killing are shown in the 11 data sets attached.

12. The results show that the "killer plasmids" (pCON4-44 and pCON4-47) could kill either 100% or very close to 100% of the exconjugants obtained from the two pathogenic bacteria under the conditions used. More details can be found in the 11 data sets attached.

13. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements are made with knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated this 15 day of June 2004.

Hideki Suzuki

QBMKE\960296.00084\5587058.1

Conjugation-dependent Killing Assay

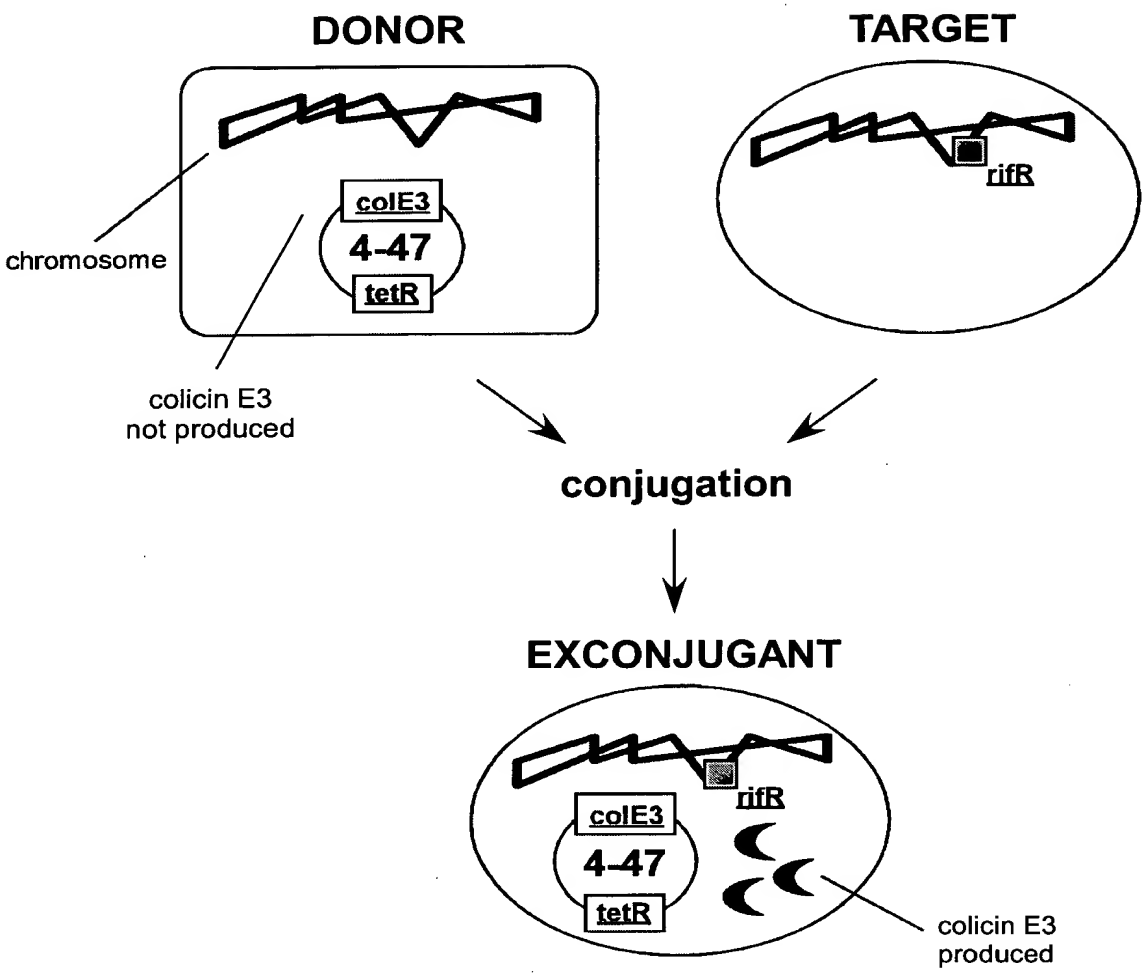


Fig. 1. Conjugation-dependent killing assay. The above diagram is an overview of the conjugation-dependent killing assays used in these experiments. The donor strain contains two plasmids, pCON4-47 and pCON1-94. The transmissible plasmid pCON4-47 contains the tetracycline-resistance gene (tetR) and a "killer" gene *colE3* (colicin E3). Bacterial cells are killed upon expression of *colE3*. In order to keep expression of *colE3* turned "off" in the donor bacteria, the donor strain carries carries the *lacIq* gene (Lac repressor protein) which efficiently represses *colE3* expression, and the *immE3* gene which confers immunity to colicin E3. The target bacterium contains a rifampicin-resistance gene (rifR) on its chromosome which makes it resistant to the antibiotic rifampicin. Target cells which receive the pCON4-47 plasmid through conjugation are called exconjugants. After the target bacterium receives pCON4-47, colicin E3 is produced and causes cell death because the target strain lacks *lacIq* and *immE3* genes. Exconjugants that are not killed can grow on plates containing both tetracycline and rifampicin.

Description of Plasmids Used in Examples

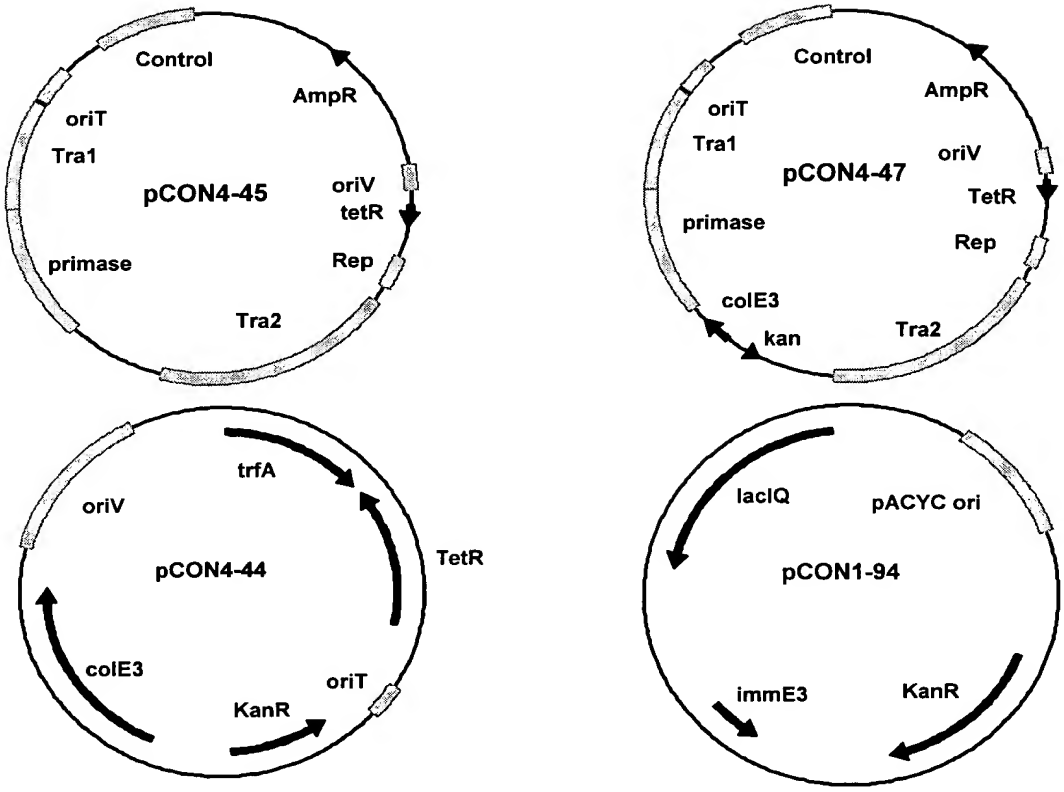


Figure 2. Description of plasmids used to kill target pathogen. pCON4-45 and pCON4-47 are self-transmissible plasmids. The latter plasmid was constructed by cloning colE3 and KanR into pCON4-45. pCON4-44 is a non-self-transmissible plasmid carrying colE3. pCON4-45 was used as a control to monitor conjugation efficiencies, and the other two plasmids were used to monitor killing effect. pCON1-94 was used as a helper plasmid to express the immunity protein to neutralize Colicin E3 within a host bacterium. This plasmid is not mobilizable, and is not transferred into a target bacterium through conjugation. Expression of the "killer gene", colE3, is repressed by abundant repressor in the donor strain in addition to the toxin-immunity protein, ImmE3. ImmE3 binds to ColE3 to prevent the toxic effect of ColE3. ImmE3 is expressed in the donor cell, therefore leaky expression of colicin E3 does not cause any detrimental effect. When colE3 is transferred into a target cell, the gene starts producing ColE3 in the target since the amount of the repressor protein and the immunity protein are not high enough. Without the repressor and ImmE3, ColE3 efficiently kill the target cell. Note that these plasmids are significantly different in their sizes; therefore sizes of the genes in this figure are not in scale.

Abbreviations used in the figure:
Rep, the region essential for replication of the plasmid from the oriV region; oriT, the region where the single-stranded DNA transfer occurs upon conjugation; primase, the region essential for synthesizing the complementary DNA strand after the single-stranded DNA is transferred into a recipient cell with conjugation; TetR, tetracycline-resistant determinant; AmpR, ampicillin-resistant determinant; KanR, kanamycin-resistant determinant; Control, the region encoding genes to control the expression of genes on the RK2 plasmid; trfA, genes encoding an essential protein to initiate replication from oriV; colE3, the gene encoding colicin E3; pACYC ori, origin of replication derived from pACYC184; lacIQ, the gene encoding the repressor to control expression of colE3; immE3, the gene encoding the immunity protein for colicin E3.

Data Set 1: Conjugation/killing experiment on flower surface (experiment 1)

Target strain: Rifampicin-resistant *Salmonella typhimurium* (also known as *Salmonella typhimurium* serotype Typhimurium)

Donor strain A: *E. coli* CON4-11c cells carrying a self-transmissible plasmid without *colE3* **(control group)**
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-45) feature: self-transmissible, selectable markers (AmpR, TetR)

Donor strain B: *E. coli* CON4-11c cells carrying a non-self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-47) feature: self-transmissible, selectable markers (KanR, AmpR, TetR), ***colE3***

Donor strain C: *E. coli* CON4-11c cells carrying a self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-44) feature: non-self-transmissible, selectable markers (KanR, TetR), ***colE3***

The plasmid carries a tetracycline-resistant determinant and the target strain carries a rifampicin-resistant determinant on the chromosome. Therefore, the target bacteria which received the plasmid (called "exconjugants") can be selected on plates containing both tetracycline and rifampicin. Note that donor and target cells cannot grow in the presence of both antibiotic markers. Viable counts of donor and target cells can be obtained by serial-dilution onto single-antibiotic plates containing tetracycline and rifampicin, respectively.

Donor strains B and C contain the antibacterial protein colicin E3 (colE3) which rapidly kills the target bacteria upon conjugative transfer of the plasmid.

For conjugation/killing experiments, the donor and target strains were grown overnight in Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C. Equal amounts of donor and recipient cells were spotted onto a flower surface for conjugation. After incubation, cells were removed, serially diluted, and spotted on LB-antibiotic plates for measuring colony forming units.

Conjugation plate (LB with Tet/Rif)

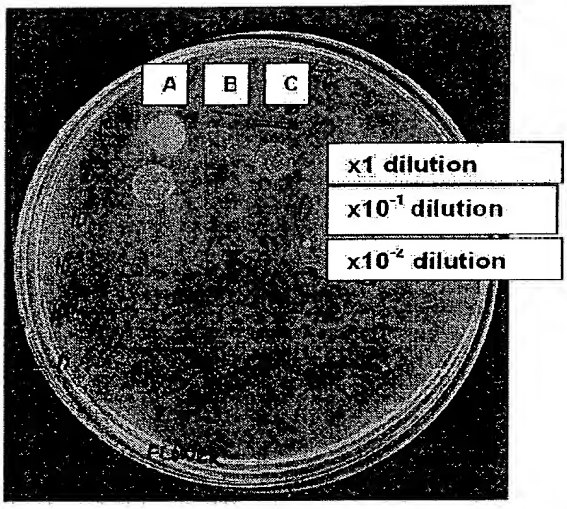
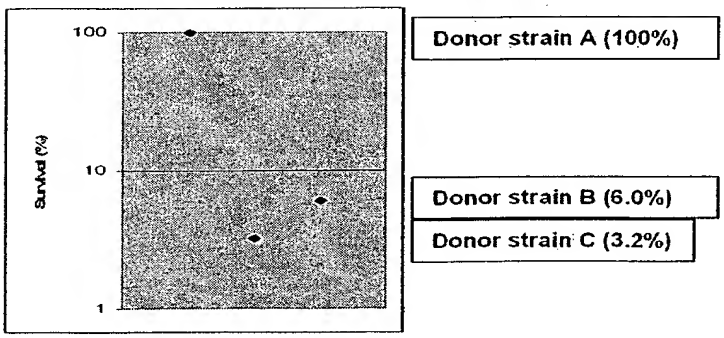


Table. Number of exconjugants counted from the plate
TNC = too numerous to count

Dilution	Donor strain (A)	Donor strain (B)	Donor strain (C)
1	TNC	27	50
10^{-1}	82	0	0
10^{-2}	0	0	0
10^{-3}	0	0	0

Comparison of survival values after conjugation (based on the data shown above)



For the control plasmid (A), 100% of target bacteria survived. When using the self-transmissible plasmid (B), 6.0% of target bacteria survived. When using the non-self-transmissible vector (C), 3.2% of the target bacteria survived.

Data Set 2: Conjugation/killing experiment on flower surface (experiment 2)

Target strain: Rifampicin-resistant *Escherichia coli* O157:H7

Donor strain A: *E. coli* CON4-11c cells carrying a self-transmissible plasmid without *colE3* **(control group)**
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn*7*] ***lacIq, immE3***
Plasmid (pCON4-45) feature: self-transmissible, selectable markers (AmpR, TetR)

Donor strain B: *E. coli* CON4-11c cells carrying a self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn*7*] ***lacIq, immE3***
Plasmid (pCON4-47) feature: self-transmissible, selectable markers (KanR, AmpR, TetR), ***colE3***

Donor strain C: *E. coli* CON4-11c cells carrying a self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn*7*] ***lacIq, immE3***
Plasmid (pCON4-44) feature: non-self-transmissible, selectable markers (KanR, TetR), ***colE3***

The plasmid carries a tetracycline-resistant determinant and the target strain carries a rifampicin-resistant determinant on the chromosome. Therefore, the target bacteria which received the plasmid (called "exconjugants") can be selected on plates containing both tetracycline and rifampicin. Note that donor and target cells cannot grow in the presence of both antibiotic markers. Viable counts of donor and target cells can be obtained by serial-dilution onto single-antibiotic plates containing tetracycline and rifampicin, respectively.

Donor strains **B** and **C** contain the antibacterial protein **colicin E3 (*colE3)*** which rapidly kills the target bacteria upon conjugative transfer of the plasmid.

For conjugation/killing experiments, the donor and target strains were grown overnight in Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C. Equal amounts of donor and recipient cells were spotted onto a **flower petal** to facilitate conjugation. After incubation, cells were removed, serially diluted, and spotted on LB-antibiotic plates for measuring colony forming units.

Conjugation plate (LB with Tet/Rif)

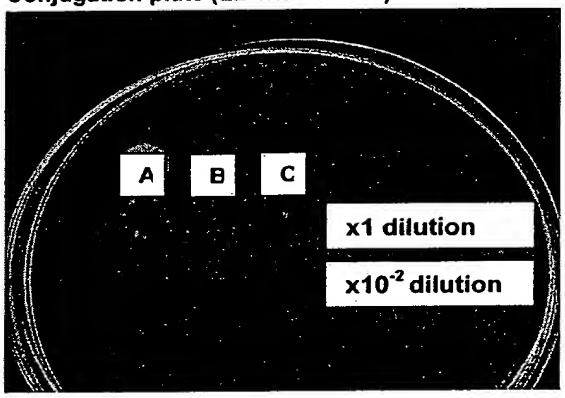
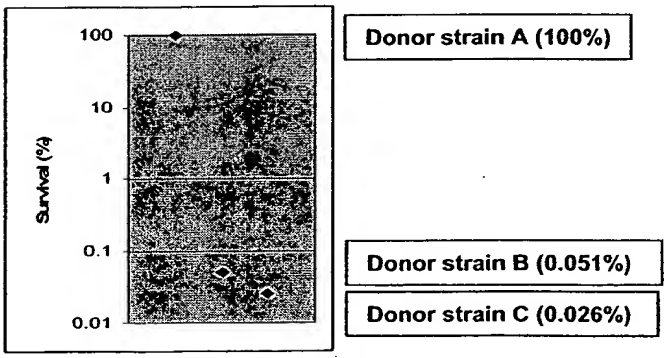


Table. Number of exconjugants counted from the plate
TNC = too numerous to count

Dilution	Donor strain (**A**)	Donor strain (**B**)	Donor strain (**C**)
1	TNC	2	1
10^{-2}	39	0	0
10^{-4}	0	0	0
10^{-6}	0	0	0

Comparison of survival values after conjugation/killing (based on the data shown above)



For the control plasmid (A), 100% of target bacteria survived. When using the self-transmissible plasmid (B), 0.051% of target bacteria survived. When using the non-self-transmissible vector (C), 0.026% of the target bacteria survived.

Data Set 3: Conjugation/killing experiment on leaf surface (experiment 1)

Target strain: Rifampicin-resistant *Salmonella typhimurium* (also known as *Salmonella enterica* serotype Typhimurium)

Donor strain A: *E. coli* CON4-11c cells carrying a self-transmissible plasmid without *colE3* **(control group)**
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-45) feature: self-transmissible, selectable markers (AmpR, TetR)

Donor strain B: *E. coli* CON4-11c cells carrying a non-self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-47) feature: self-transmissible, selectable markers (KanR, AmpR, TetR), ***colE3***

Donor strain C: *E. coli* CON4-11c cells carrying a self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-44) feature: non-self-transmissible, selectable markers (KanR, TetR), ***colE3***

The plasmid carries a tetracycline-resistant determinant and the target strain carries a rifampicin-resistant determinant on the chromosome. Therefore, the target bacteria which received the plasmid (called "exconjugants") can be selected on plates containing both tetracycline and rifampicin. Note that donor and target cells cannot grow in the presence of both antibiotic markers. Viable counts of donor and target cells can be obtained by serial-dilution onto single-antibiotic plates containing tetracycline and rifampicin, respectively.

Donor strains B and C contain the antibacterial protein colicin E3 (colE3) which rapidly kills the target bacteria upon conjugative transfer of the plasmid.

For conjugation/killing experiments, the donor and target strains were grown overnight in Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C. Equal amounts of donor and recipient cells were spotted onto a leaf surface to facilitate conjugation. After incubation, cells were removed, serially diluted, and spotted on LB-antibiotic plates for measuring colony forming units.

Conjugation plate (LB with Tet/Rif)

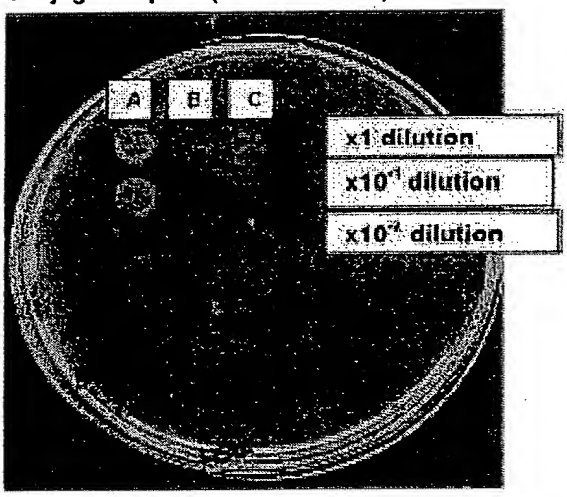
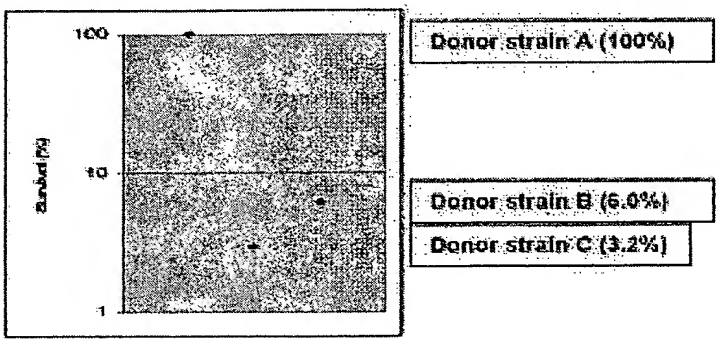


Table. Number of exconjugants counted from the plate
TNC = too numerous to count

Dilution	Donor strain (A)	Donor strain (B)	Donor strain (C)
1	TNC	24	49
10^{-1}	78	0	0
10^{-2}	0	0	0
10^{-3}	0	0	0

Comparison of survival values after conjugation (based on the data shown above)



For the control plasmid (A), 100% of target bacteria survived. When using the self-transmissible plasmid (B), 6.0% of target bacteria survived. When using the non-self-transmissible vector (C), 3.2% of the target bacteria survived.

Data Set 4: Conjugation/killing experiment on leaf surface(experiment 2)

Target strain: Rifampicin-resistant *Escherichia coli* O157:H7

Donor strain A: *E. coli* CON4-11c cells carrying a self-transmissible plasmid without *colE3* (**control group**)
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn*7*] ***lacIq, immE3***
Plasmid (pCON4-45) feature: self-transmissible, selectable markers (AmpR, TetR)

Donor strain B: *E. coli* CON4-11c cells carrying a non-self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn*7*] ***lacIq, immE3***
Plasmid (pCON4-47) feature: self-transmissible, selectable markers (KanR, AmpR,TetR), ***colE3***

Donor strain C: *E. coli* CON4-11c cells carrying a self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn*7*] ***lacIq, immE3***
Plasmid (pCON4-44) feature: non-self-transmissible, selectable markers (KanR, TetR), ***colE3***

The plasmid carries a tetracycline-resistant determinant and the target strain carries a rifampicin-resistant determinant on the chromosome. Therefore, the target bacteria which received the plasmid (called "exconjugants") can be selected on plates containing both tetracycline and rifampicin. Note that donor and target cells cannot grow in the presence of both antibiotic markers. Viable counts of donor and target cells can be obtained by serial-dilution onto single-antibiotic plates containing tetracycline and rifampicin, respectively.

Donor strains **B** and **C** contain the antibacterial protein **colicin E3 (*colE3)*** which rapidly kills the target bacteria upon conjugative transfer of the plasmid.

For conjugation/killing experiments, the donor and target strains were grown overnight in Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C. Equal amounts of donor and recipient cells were spotted onto a **leaf surface** to facilitate conjugation. After incubation, cells were removed, serially diluted, and spotted on LB-antibiotic plates for measuring colony forming units.

Conjugation plate (LB with Tet/Rif)

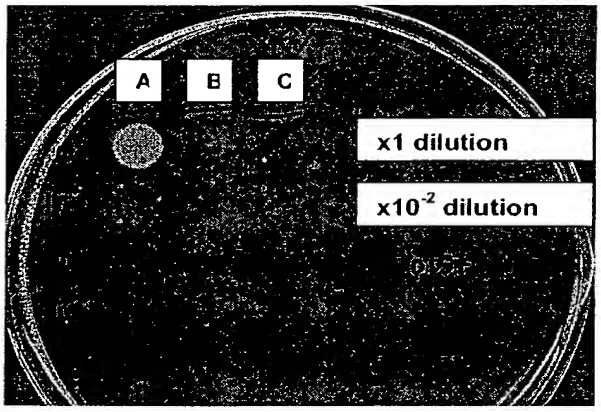
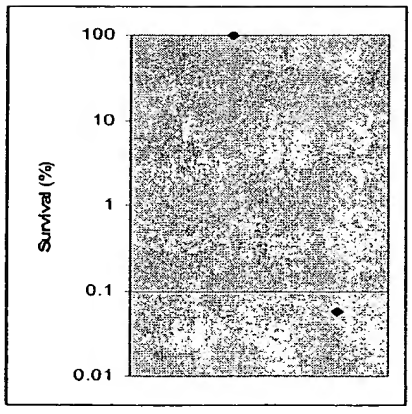


Table. **Number of exconjugants counted from the plate**
TNC = too numerous to count

Dilution	Donor strain (**A**)	Donor strain (**B**)	Donor strain (**C**)
1	TNC	0	1
10^{-2}	18	0	0
10^{-4}	0	0	0
10^{-6}	0	0	0

Survival values after conjugation (based on the data shown above)



Donor strain A (100%)

Donor strain B (0.056%)

Donor strain C (0%)

For the control plasmid (A), 100% of target bacteria survived. When using the self-transmissible plasmid (B), 0.056% of target bacteria survived. When using the non-self-transmissible vector (C), 0.0% of the target bacteria survived.

Data Set 5: Conjugation/killing experiment on meat surface (experiment 1)

Target strain: Rifampicin-resistant *Salmonella typhimurium* (also known as *Salmonella enterica* serotype Typhimurium)

Donor strain A: *E. coli* CON4-11c cells carrying a self-transmissible plasmid without *colE3* **(control group)**
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-45) feature: self-transmissible, selectable markers (AmpR, TetR)

Donor strain B: *E. coli* CON4-11c cells carrying a non-self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-47) feature: self-transmissible, selectable markers (KanR, AmpR, TetR), ***colE3***

Donor strain C: *E. coli* CON4-11c cells carrying a self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-44) feature: non-self-transmissible, selectable markers (KanR, TetR), ***colE3***

The plasmid carries a tetracycline-resistant determinant and the target strain carries a rifampicin-resistant determinant on the chromosome. Therefore, the target bacteria which received the plasmid (called "exconjugants") can be selected on plates containing both tetracycline and rifampicin. Note that donor and target cells cannot grow in the presence of both antibiotic markers. Viable counts of donor and target cells can be obtained by serial-dilution onto single-antibiotic plates containing tetracycline and rifampicin, respectively.

Donor strains B and C contain the antibacterial protein colicin E3 (colE3) which rapidly kills the target bacteria upon conjugative transfer of the plasmid.

For conjugation/killing experiments, the donor and target strains were grown overnight in Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C. Equal amounts of donor and recipient cells were spotted onto a meat surface to facilitate conjugation. After incubation, cells were removed, serially diluted, and spotted on LB-antibiotic plates for measuring colony forming units.

Conjugation plate (LB with Tet/Rif)

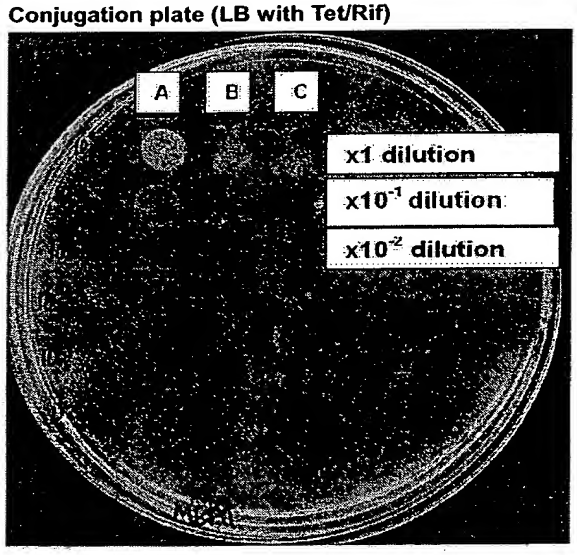


Table. Number of exconjugants counted from the plate
TNC = too numerous to count

Dilution	Donor strain (A)	Donor strain (B)	Donor strain (C)
1	TNC	0	0
10^{-1}	49	0	0
10^{-2}	0	0	0
10^{-3}	0	0	0

Comparison of survival values after conjugation (based on the data shown above)

No surviving colonies were detected after conjugation/killing with the self-transmissible or non-self-transmissible plasmid.

Data Set 6: Conjugation/killing experiment on meat surface (experiment 2)

Target strain: Rifampicin-resistant *Escherichia coli* O157:H7

Donor strain A: *E. coli* CON4-11c cells carrying a self-transmissible plasmid without *colE3* **(control group)**
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-45) feature: self-transmissible, selectable markers (AmpR, TetR)

Donor strain B: *E. coli* CON4-11c cells carrying a non-self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-47) feature: self-transmissible, selectable markers (KanR, AmpR, TetR), ***colE3***

Donor strain C: *E. coli* CON4-11c cells carrying a self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-44) feature: non-self-transmissible, selectable markers (KanR, TetR), ***colE3***

The plasmid carries a tetracycline-resistant determinant and the target strain carries a rifampicin-resistant determinant on the chromosome. Therefore, the target bacteria which received the plasmid (called "exconjugants") can be selected on plates containing both tetracycline and rifampicin. Note that donor and target cells cannot grow in the presence of both antibiotic markers. Viable counts of donor and target cells can be obtained by serial-dilution onto single-antibiotic plates containing tetracycline and rifampicin, respectively.

Donor strains B and C contain the antibacterial protein colicin E3 (colE3) which rapidly kills the target bacteria upon conjugative transfer of the plasmid.

For conjugation/killing experiments, the donor and target strains were grown overnight in Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C. Equal amounts of donor and recipient cells were onto the surface of meat for conjugation. After incubation, cells were removed, serially diluted, and spotted on LB-antibiotic plates for measuring colony forming units.

Conjugation plate (LB with Tet/Rif)

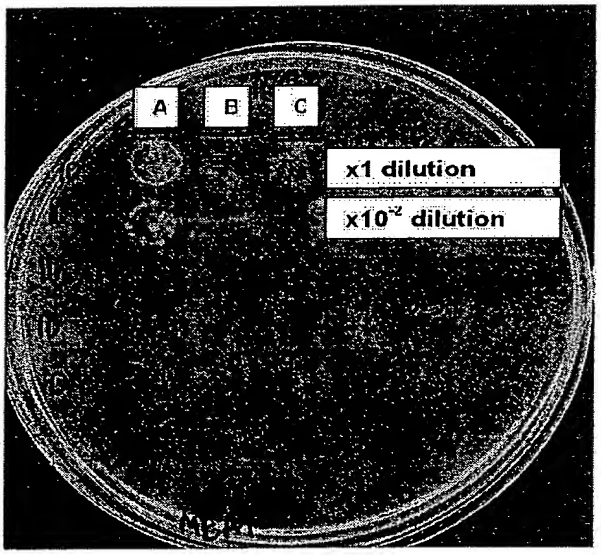
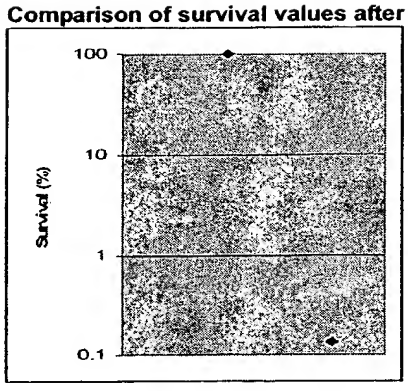


Table. Number of exconjugants counted from the plate
TNC = too numerous to count

Dilution	Donor strain (A)	Donor strain (B)	Donor strain (C)
1	TNC	0	1
10^{-2}	18	0	0
10^{-4}	0	0	0
10^{-6}	0	0	0

Comparison of survival values after conjugation (based on the data shown above)



Donor strain A (100%)

Donor strain B (0.133%)

Donor strain C (0%)

For the control plasmid (A), 100% of target bacteria survived. When using the self-transmissible plasmid (B), 0.133% of target bacteria survived. When using the non-self-transmissible vector (C), 0% of the target bacteria survived.

Data Set 7: Conjugation/killing experiment on a potato surface (experiment 1)

Target strain: Rifampicin-resistant *Salmonella typhimurium* (also known as *Salmonella enterica* serotype Typhimurium)

Donor strain A: *E. coli* CON4-11c cells carrying a self-transmissible plasmid without *colE3* **(control group)**
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-45) feature: self-transmissible, selectable markers (AmpR, TetR)

Donor strain B: *E. coli* CON4-11c cells carrying a non-self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-44) feature: non-self-transmissible, selectable markers (KanR, TetR), ***colE3***

Donor strain C: *E. coli* CON4-11c cells carrying a self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-47) feature: self-transmissible, selectable markers (KanR, AmpR, TetR), ***colE3***

The plasmid carries a tetracycline-resistant determinant and the target strain carries a rifampicin-resistant determinant on the chromosome. Therefore, the target bacteria which received the plasmid (called "exconjugants") can be selected on plates containing both tetracycline and rifampicin. Note that donor and target cells cannot grow in the presence of both antibiotic markers. Viable counts of donor and target cells can be obtained by serial-dilution onto single-antibiotic plates containing tetracycline and rifampicin, respectively.

Donor strains B and C contain the antibacterial protein colicin E3 (colE3) which rapidly kills the target bacteria upon conjugative transfer of the plasmid.

For conjugation/killing experiments, the donor and target strains were grown overnight in Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C. Equal amounts of donor and recipient cells were spotted onto a sliced potato surface for conjugation. After incubation, cells were removed, serially diluted, and spotted on LB-antibiotic plates for measuring colony forming units.

Conjugation plate (LB with Tet/Rif)

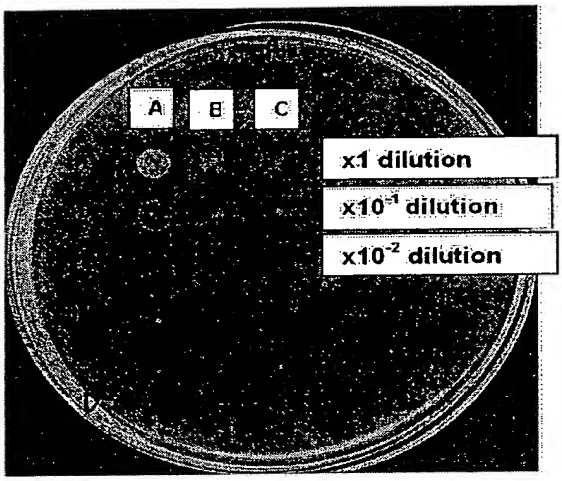


Table. Number of exconjugants counted from the plate
TNC = too numerous to count

Dilution	Donor strain (A)	Donor strain (B)	Donor strain (C)
1	TNC	0	0
10^{-1}	20	0	0
10^{-2}	1	0	0
10^{-3}	0	0	0

Comparison of survival values after conjugation (based on the data shown above)

No surviving colonies were detected after conjugation/killing with the non-self-transmissible or self-transmissible plasmid.

Data Set 8: Conjugation/killing experiment on a potato surface (experiment 2)

Target strain: Rifampicin-resistant *Escherichia coli* O157:H7

Donor strain A: *E. coli* CON4-11c cells carrying a self-transmissible plasmid without *colE3* **(control group)**
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-45) feature: self-transmissible, selectable markers (AmpR, TetR)

Donor strain B: *E. coli* CON4-11c cells carrying a non-self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-47) feature: self-transmissible, selectable markers (KanR, AmpR, TetR), ***colE3***

Donor strain C: *E. coli* CON4-11c cells carrying a self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-44) feature: non-self-transmissible, selectable markers (KanR, TetR), ***colE3***

The plasmid carries a tetracycline-resistant determinant and the target strain carries a rifampicin-resistant determinant on the chromosome. Therefore, the target bacteria which received the plasmid (called "exconjugants") can be selected on plates containing both tetracycline and rifampicin. Note that donor and target cells cannot grow in the presence of both antibiotic markers. Viable counts of donor and target cells can be obtained by serial-dilution onto single-antibiotic plates containing tetracycline and rifampicin, respectively.

Donor strains B and C contain the antibacterial protein colicin E3 (colE3) which rapidly kills the target bacteria upon conjugative transfer of the plasmid.

For conjugation/killing experiments, the donor and target strains were grown overnight in Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C. Equal amounts of donor and recipient cells were spotted onto a sliced potato surface for conjugation. After incubation, cells were removed, serially diluted, and spotted on LB-antibiotic plates for measuring colony forming units.

Conjugation plate (LB with Tet/Rif)

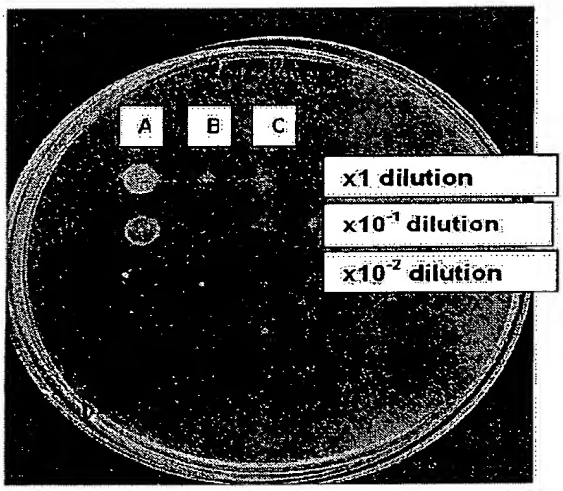


Table. Number of exconjugants counted from the plate
TNC = too numerous to count

Dilution	Donor strain (A)	Donor strain (B)	Donor strain (C)
1	TNC	0	0
10^{-1}	75	0	0
10^{-2}	0	0	0
10^{-3}	0	0	0

Comparison of survival values after conjugation (based on the data shown above)

No surviving colonies were detected after conjugation/killing with the non-self-transmissible or self-transmissible plasmid.

Data Set 9: Conjugation/killing experiment in blood plasma

Target strain: Rifampicin-resistant *Escherichia coli* O157:H7

Donor strain A: *E. coli* CON4-11c cells carrying a self-transmissible plasmid without *colE3* **(control group)**
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-45) feature: self-transmissible, selectable markers (AmpR, TetR)

Donor strain B: *E. coli* CON4-11c cells carrying a non-self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-47) feature: self-transmissible, selectable markers (KanR, AmpR, TetR), ***colE3***

Donor strain C: *E. coli* CON4-11c cells carrying a self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-44) feature: non-self-transmissible, selectable markers (KanR, TetR), ***colE3***

The plasmid carries a tetracycline-resistant determinant and the target strain carries a rifampicin-resistant determinant on the chromosome. Therefore, the target bacteria which received the plasmid (called "exconjugants") can be selected on plates containing both tetracycline and rifampicin. Note that donor and target cells cannot grow in the presence of both antibiotic markers. Viable counts of donor and target cells can be obtained by serial-dilution onto single-antibiotic plates containing tetracycline and rifampicin, respectively.

Donor strains B and C contain the antibacterial protein colicin E3 (colE3) which rapidly kills the target bacteria upon conjugative transfer of the plasmid.

For conjugation/killing experiments, the donor and target strains were grown overnight in Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C. Equal amounts of donor and recipient cells were diluted into blood plasma for conjugation. After incubation, cells were removed, serially diluted, and spotted on LB-antibiotic plates for measuring colony forming units.

Conjugation plate (LB with Tet/Rif)

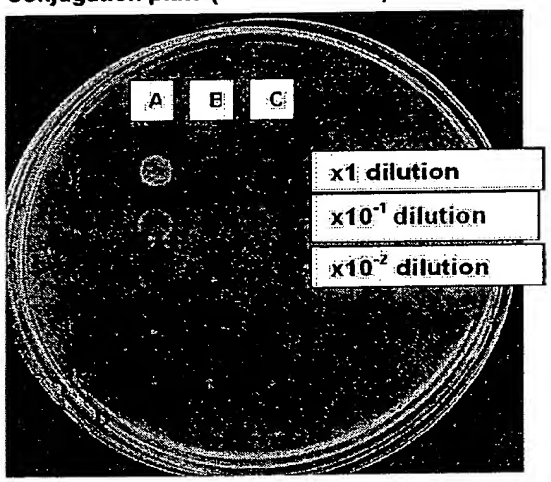


Table. Number of exconjugants counted from the plate
TNC = too numerous to count

Dilution	Donor strain (A)	Donor strain (B)	Donor strain (C)
1	TNC	0	0
10^{-1}	45	0	0
10^{-2}	0	0	0
10^{-3}	0	0	0

Comparison of survival values after conjugation (based on the data shown above)

No surviving colonies were detected after conjugation/killing with the non-self-transmissible or self-transmissible plasmid.

Data Set 10: Conjugation/killing experiment in human urine (experiment 1)

Target strain: Rifampicin-resistant *Salmonella typhimurium* (also known as *Salmonella enterica* serotype Typhimurium)

Donor strain A: *E. coli* CON4-11c cells carrying a self-transmissible plasmid without *colE3* **(control group)**
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-45) feature: self-transmissible, selectable markers (AmpR, TetR)

Donor strain B: *E. coli* CON4-11c cells carrying a non-self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-44) feature: non-self-transmissible, selectable markers (KanR, TetR), ***colE3***

Donor strain C: *E. coli* CON4-11c cells carrying a self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-47) feature: self-transmissible, selectable markers (KanR, AmpR, TetR), ***colE3***

The plasmid carries a tetracycline-resistant determinant and the target strain carries a rifampicin-resistant determinant on the chromosome. Therefore, the target bacteria which received the plasmid (called "exconjugants") can be selected on plates containing both tetracycline and rifampicin. Note that donor and target cells cannot grow in the presence of both antibiotic markers. Viable counts of donor and target cells can be obtained by serial-dilution onto single-antibiotic plates containing tetracycline and rifampicin, respectively.

Donor strains B and C contain the antibacterial protein colicin E3 (colE3) which rapidly kills the target bacteria upon conjugative transfer of the plasmid.

For conjugation/killing experiments, the donor and target strains were grown overnight in Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C. Equal amounts of donor and recipient cells were diluted into human urine for conjugation. After incubation, cells were removed, serially diluted, and spotted on LB-antibiotic plates for measuring colony forming units.

Conjugation plate (LB with Tet/Rif)

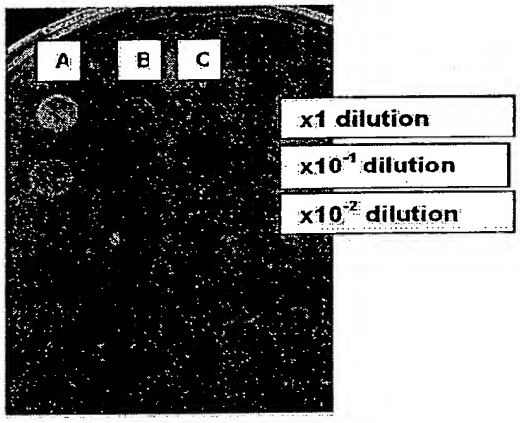


Table. Number of exconjugants counted from the plate
TNC = too numerous to count

Dilution	Donor strain (A)	Donor strain (B)	Donor strain (C)
1	TNTC	0	0
10^{-1}	220	0	0
10^{-2}	2	0	0
10^{-3}	0	0	0

Comparison of survival values after conjugation (based on the data shown above)

No surviving colonies were detected after conjugation/killing with the non-self-transmissible or self-transmissible plasmid.

Data Set 11: Conjugation/killing experiment in human urine (experiment 2)

Target strain: Rifampicin-resistant *Escherichia coli* O157:H7

Donor strain A: *E. coli* CON4-11c cells carrying a self-transmissible plasmid without *colE3* **(control group)**
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-45) feature: self-transmissible, selectable markers (AmpR, TetR)

Donor strain B: *E. coli* CON4-11c cells carrying a non-self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-47) feature: self-transmissible, selectable markers (KanR, AmpR, TetR), ***colE3***

Donor strain C: *E. coli* CON4-11c cells carrying a self-transmissible plasmid with *colE3*
CON4-11c: a derivative of *E. coli* S17-1, C600 [RK2-2-Tet::Mu Kan::Tn7] ***lacIq, immE3***
Plasmid (pCON4-44) feature: non-self-transmissible, selectable markers (KanR, TetR), ***colE3***

The plasmid carries a tetracycline-resistant determinant and the target strain carries a rifampicin-resistant determinant on the chromosome. Therefore, the target bacteria which received the plasmid (called "exconjugants") can be selected on plates containing both tetracycline and rifampicin. Note that donor and target cells cannot grow in the presence of both antibiotic markers. Viable counts of donor and target cells can be obtained by serial-dilution onto single-antibiotic plates containing tetracycline and rifampicin, respectively.

Donor strains B and C contain the antibacterial protein colicin E3 (colE3) which rapidly kills the target bacteria upon conjugative transfer of the plasmid.

For conjugation/killing experiments, the donor and target strains were grown overnight in Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C. Equal amounts of donor and recipient cells were diluted into human urine for conjugation. After incubation, cells were removed, serially diluted, and spotted on LB-antibiotic plates for measuring colony forming units.

Conjugation plate (LB with Tet/Rif)

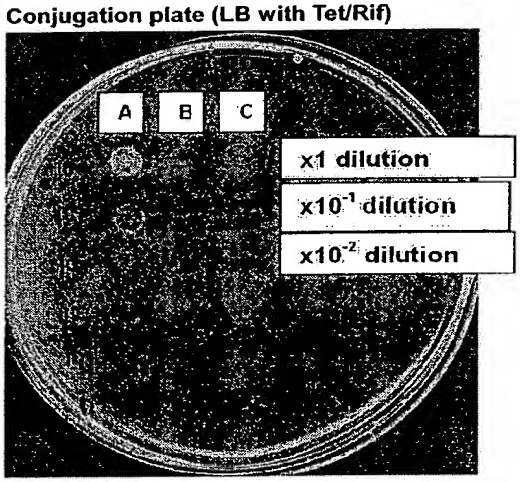


Table. Number of exconjugants counted from the plate
TNC = too numerous to count

Dilution	Donor strain (**A**)	Donor strain (**B**)	Donor strain (**C**)
1	TNC	0	0
10^{-1}	33	0	0
10^{-2}	0	0	0
10^{-3}	0	0	0

Comparison of survival values after conjugation (based on the data shown above)

No surviving colonies were detected after conjugation/killing with the non-self-transmissible or self-transmissible plasmid.

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A Novel Suicide Vector and Its Use in Construction of Insertion Mutations: Osmoregulation of Outer Membrane Proteins and Virulence Determinants in *Vibrio cholerae* Requires *toxR*

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The *toxR* gene of *Vibrio cholerae* encodes a transmembrane, DNA-binding protein that activates transcription of the cholera toxin operon and a gene (*tcpA*) for the major subunit of a pilus colonization factor. We constructed site-directed insertion mutations in the *toxR* gene by a novel method employing the chromosomal integration of a mobilizable suicide plasmid containing a portion of the *toxR* coding sequence. Mutants containing these new *toxR* alleles had an altered outer membrane protein profile, suggesting that two major outer membrane proteins (OmpT and OmpU) might be under the control of *toxR*. Physiological studies indicated that varying the concentration of the amino acids asparagine, arginine, glutamate, and serine caused coordinate changes in the expression of cholera toxin, TcpA, OmpT, and OmpU. Changes in the osmolarity of a tryptone-based medium also produced coordinate changes in the expression of these proteins. Other environmental signals (temperature and pH) had a more pronounced effect on the expression of cholera toxin and TcpA than they did on the outer membrane proteins. These results suggest that certain environmental signals (i.e., osmolarity and the presence of amino acids) are tightly coupled to the expression of *toxR*-regulated proteins and therefore may be signals that are directly sensed by the ToxR protein.

Little is known about the in vivo environmental signals that control expression of bacterial virulence determinants. The effects of nutritional and physical parameters on the production of virulence factors in laboratory media reflect the existence of regulatory mechanisms that may help the microbe determine when it is appropriate to express these rather specialized properties. This regulation presumably allows the organism to avoid the metabolic drain of producing toxins, colonization factors, capsules, and other virulence-enhancing proteins in environments where their action is not needed. A wide spectrum of compounds and growth conditions have been implicated in the regulation of virulence properties, including iron (2, 23), divalent cations (24, 25, 29, 33), atmospheric gases (26), temperature (14), and even complex organic molecules like nicotinic acid (33) and phenolic compounds (28). Understanding the signals and mechanisms that are involved in the control of virulence gene expression might someday lead to applications in vaccine development and chemotherapy of bacterial infections.

Vibrio cholerae is a gram-negative bacterium that causes a severe diarrheal disease by colonizing the upper intestine of humans and elaborating a protein exotoxin (1). Cholera toxin is a multimeric protein composed of two types of subunits, A and B, that are encoded by the genes *ctxA* and *ctxB*, respectively (18). The *ctxA* and *ctxB* genes form an operon that is positively regulated at the transcriptional level by the product of the *toxR* gene (20–22). Recently, we have shown that the *toxR* gene regulates not only the *ctx* operon but also the gene (*tcpA*) for the major subunit of a toxin-coregulated pilus colonization factor called TCP (31). Thus, the *toxR* gene product plays a central role in controlling the expression of multiple virulence properties of *V. cholerae*.

The *toxR* gene encodes a 32,527-dalton transmembrane, DNA-binding protein that may have the ability to sense a variety of environmental signals that include osmolarity, pH, temperature, and the presence of certain amino acids (22). Alteration of the *toxR* gene in *V. cholerae* through the construction of specific missense and deletion mutations might provide a means of defining which domains of the ToxR protein are involved in sensory functions, as well as help in the identification of additional *V. cholerae* gene products that might be under *toxR* control.

Previously isolated *toxR* mutants (6, 17) are not ideal for this type of analysis because they were isolated after treatment with *N*-methyl-*N'*-nitrosoguanidine, a mutagen known to induce a high frequency of secondary mutations. Here we report the construction of site-directed *toxR* insertion mutations constructed by a novel method employing the chromosomal integration of a mobilizable suicide plasmid containing a portion of the *toxR* coding sequence. Characterization of mutant and parental strains suggests that ToxR regulates the expression of toxin, TCP, and outer membrane proteins in response to several environmental signals, of which osmolarity and the presence of amino acids seem the most important.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are described in Table 1. Bacterial strains were maintained at −70°C in LB medium containing 25% (vol/vol) glycerol (19). LB, M9 minimal, and CYE media were prepared as described previously (16, 19). M9 minimal medium contained 0.4% glycerol as the carbon source and was supplemented as indicated with 25 mM asparagine, arginine, glutamate, and serine. The antibiotics ampicillin and kanamycin were used at 100 and 45 μg/ml,

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TABLE 1. Strains and plasmids used in this study

Strain, plasmid, or bacteriophage	Genotype or phenotype	Reference or source
V. cholerae		
569B Sm	*str*	16
O395 Sm	*str*	18, 31
CA401 Sm	*str*	31
M13 Sm	*tox-2 str*	6
569B-55	*str toxR*::pVM55 Ap	This study
O395-55	*str toxR*::pVM55 Ap	This study
CA401-55	*str toxR*::pVM55 Ap	This study
O395-12	*str toxR*::pJM703.12 Ap	This study
RT110.21	*str tcpA21*::Tn*phoA* Km	31
E. coli		
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km	27
SY327	Δ(*lac pro*) *argE*(Am) *rif nalA recA56*	20
Plasmids		
pVM7	*oriE1 toxR*$^+$ Ap	20
pRK703	*oriE1 oriR6K* Ap	10
pSUP201-1	*oriE1 mobRP4* Ap Cm	27
pJM703	*oriR6K* Ap	This study
pJM703.1	*oriR6K mobRP4* Ap	This study
pVM55	pJM703.1::*Eco*RI-*Hpa*I (*toxR*) Ap	This study
pJM703.12	pJM703.1::*Eco*RI-*Nru*I (*toxR*) Ap	This study
Bacteriophage λ *pir*	*pir*RK6	10

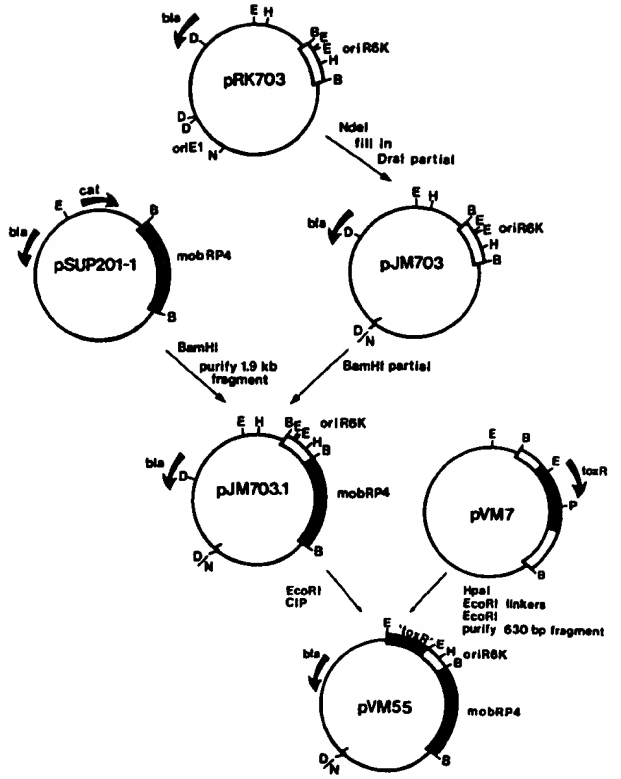

FIG. 1. Construction of pJM703.1 and pVM55. The following letters are used to denote different restriction enzyme sites on the maps: B, *Bam*HI; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; N, *Nde*I; P, *Hpa*I. CIP, Calf intestinal alkaline phosphatase.

respectively. In all experiments, except those for the preparation of outer membranes, 2-ml cultures in test tubes (13 by 100 mm) were used and were incubated with constant mixing at 30 rpm on a roller incubator (model TC-7; New Brunswick Scientific Co., Inc., Edison, N.J.) until the early stationary phase (usually 18 to 24 h at 30°C).

Nucleic acid preparation and analysis. DNA fragments were purified from agarose gels by the freeze-squeeze technique (32). *V. cholerae* chromosomal DNA was purified, and Southern blot analysis was performed as described previously (15). The *toxR* probe was plasmid pVM7 (20), which was labeled by a nick-translation procedure (13). DNA restriction enzymes and phage T4 DNA ligase were purchased from New England BioLabs, Inc. (Beverly, Mass.). Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

Construction of pJM703.1. Plasmid pJM703.1 was constructed as follows (Fig. 1). pRK703, a derivative of pBR322 that has a 420-base-pair (bp) *Bam*HI fragment containing the origin of replication of plasmid R6K (10) inserted in the *Bam*HI site, was digested with *Nde*I, and the overhanging ends were filled in with Klenow fragment. The plasmid was then partially digested with *Dra*I, ligated, and used to transform competent cells of a λ *pir* lysogen of *Escherichia coli* SY327. The *pir* gene encodes a protein which is required for function of the R6K origin (10). Ap^r transformants were selected, and plasmid DNA was purified and screened for the ability to replicate in SY327. Of 24 plasmids screened, one, pJM703, was able to replicate in SY327 λ *pir* but not in SY327; this plasmid (pJM703) has the R6K origin of replication and has a deletion of the pBR322 origin of replication. pJM703 was then partially digested with *Bam*HI and ligated to a purified 1.9-kilobase (kb) *Bam*HI fragment from pSUP201-1, which carries the *mob* region of pRP4 (27). The ligated plasmids were used to transform competent cells of SY327 λ *pir*. Restriction enzyme analysis identified a plasmid (pJM703.1) that carried the *mob* insert.

Construction of derivatives of plasmid pJM703.1 carrying *toxR* internal sequences. Plasmid pVM55 was constructed by first digesting the *toxR* plasmid pVM7 with *Hpa*I, which cuts internally in the *toxR* coding sequence 630 bp downstream of an internal *Eco*RI site (22). *Hpa*I-digested pVM7 was ligated to *Eco*RI linkers, digested with *Eco*RI, and electrophoresed in a 1.0% agarose gel. The 630-bp *toxR* internal fragment was purified from an agarose gel slice and ligated to pJM703.1, which was previously digested with *Eco*RI and treated with calf alkaline intestinal phosphatase. The ligated DNA was then used to transform competent cells of strain SY327 λ *pir*; Ap^r transformants were selected and plasmid DNA was analyzed with restriction endonucleases to identify a plasmid (pVM55) carrying the correct insert (Fig. 1). Plasmid pJM703.12 was constructed in the same way as pVM55, but contained a 237-bp *Eco*RI-*Nru*I *toxR* fragment (22). Both pVM55 and pJM703.12 were transformed into a λ *pir* lysogen of strain SM10 (27). SM10 λ *pir* can mobilize pJM703.1 derivatives into *V. cholerae* because it carries a derivative of plasmid RP4 integrated in the bacterial chromosome. This derivative can provide conjugative functions in *trans* to the *mob* site on pJM703.1 but is rarely transferred itself to the recipient strain (27).

Toxin assay. Cholera toxin was measured in culture supernatant fluids by GM1 ganglioside-dependent enzyme-linked immunosorbent assay (7).

Fractionation of *V. cholerae* cells. Large-scale cell fraction-

ation for the preparation of outer membranes was performed essentially as described previously (9). Cells from 250-ml CYE cultures grown at 30°C with aeration were pelleted by centrifugation at 10,000 × *g* for 20 min. The pellet was suspended in 100 ml of HMNS buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4], 10 mM $MgCl_2$, 150 mM NaCl, 5% sucrose), centrifuged again at 10,000 × *g* for 15 min, and suspended in 30 ml of cold HMNS buffer. The washed cells were then broken by passing them through a French press at 15,000 lb/in^2. The unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min. The supernatant was then centrifuged in a Beckman Ti50 rotor at 12,000 rpm for 90 min at 5°C. The pellet (envelope fraction) and the supernatant (crude cytosol fraction) were then fractionated further. Contaminating membranes were removed from the crude cytosol fraction by incubating it at 30°C for 2 h and then centrifuging it in a Ti50 rotor at 16,000 rpm for 90 min at 5°C. The resulting supernatant was designated the cytosolic fraction. The envelope fraction was suspended in 20 ml of HMNS buffer, and 4 ml of 10% Triton X-100–10 mM $MgCl_2$ was added. After incubation at 25°C for 20 min and centrifugation in the Ti50 rotor at 40,000 rpm for 90 min at 5°C, the supernatant (Triton X-100-soluble fraction) containing inner membrane proteins was collected. The pellet (Triton X-100-insoluble fraction) containing outer membrane proteins was suspended in 24 ml of HMNS. All fractions were stored at −20°C.

Polyacrylamide gel electrophoresis. Analyses of total cell protein and cell envelope fractions were performed by polyacrylamide gel electrophoresis (PAGE) in 12.5% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) followed by staining with Coomassie brilliant blue as described previously (31).

RESULTS

Construction of *toxR* insertion mutations. Construction of *V. cholerae* strains carrying chromosomal deletions and insertions in the *toxR* gene was found to be technically difficult by previously described chromosomal marker exchange methods (18). We therefore developed a method for construction of *toxR* mutations by insertion of a plasmid containing part of the *toxR* gene into the chromosomal copy of *toxR* by homologous recombination.

To do this we first constructed pJM703, a derivative of pBR322 that has a deletion of the pBR322 origin of replication (*oriE1*) but that carries, instead, a cloned fragment containing the origin of replication of plasmid R6K (10). The R6K origin of replication (*oriR6K*) requires for its function a protein called π, which is encoded by the *pir* gene, which in our experiments was supplied in *trans* in *Escherichia coli* by a prophage (λ *pir*), a derivative of phage λ carrying a cloned copy of the *pir* gene (10). A derivative of pJM703 was then constructed (pJM703.1) that contains a 1.9-kb *Bam*HI fragment encoding the *mob* region of RP4 (27). Plasmid pJM703.1 can be mobilized into *V. cholerae* by transfer functions provided by a derivative of RP4 integrated in the chromosome of *E. coli* SM10 (27), but it is unable to replicate in *V. cholerae* because *V. cholerae* does not provide the essential π protein function.

Insertion mutations in the chromosomal *toxR* gene were isolated by first subcloning DNA fragments carrying sequences internal to the *toxR* coding sequence into pJM703.1. Two such plasmids were constructed (pVM55 and pJM703.12; see above) and were then mobilized into *V. cholerae*. Because these pJM703.1 derivatives cannot replicate in *V. cholerae*, Ap^r transconjugants should contain the mobilized plasmid integrated into the genome by homologous recombination between the *toxR* gene on the chromosome and the cloned sequences present on the plasmids.

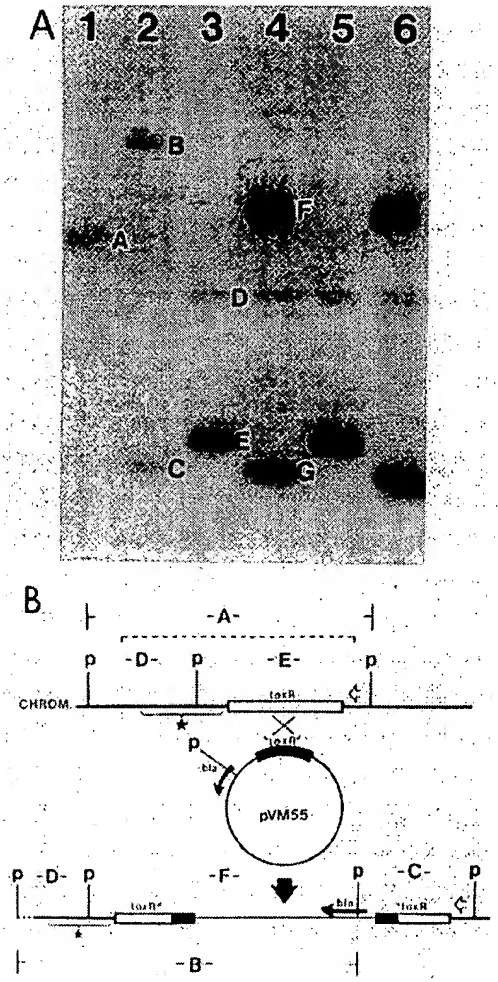

FIG. 2. Southern blot analysis of pVM55 integration events. (A) Chromosomal DNA samples from the strains indicated below were digested with *Pst*I, electrophoresed in an agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pVM7. Bands appearing on the autoradiograph are labeled with letters that correspond to the fragments indicated on the schematic diagram given in panel B. Lanes: 1, 569B; 2, 569B-55; 3, O395; 4, O395-55; 5, CA401; 6, CA401-55. (B) The pVM7 probe carries sequences derived from strain 569B (20) that correspond to the dotted bracket above the top line of panel B. Strain 569B carries a chromosomal deletion mutation (brace with a star) downstream from *toxR* (21) that removes a *Pst*I restriction enzyme site (p). Strains O395 and CA401 do not have this deletion mutation (21). The solid box on pVM55 represents an internal fragment of the *toxR* gene corresponding to the *Eco*RI-*Hpa*I fragment from pVM7. Homologous recombination (×) integrates pVM55 into the chromosome to give the fragments indicated on the diagram. Fragment C is the same as fragment G in this analysis.

This was shown to be the case for the plasmid pVM55 by Southern blot hybridization of representative transconjugants of *V. cholerae* 569B, O395, and CA401 (Fig. 2). Integration of pVM55 disrupted the *toxR* gene (Fig. 2B), an event that can be followed by loss of the original chromosomal fragment carrying *toxR* (fragment A for strain 569B)

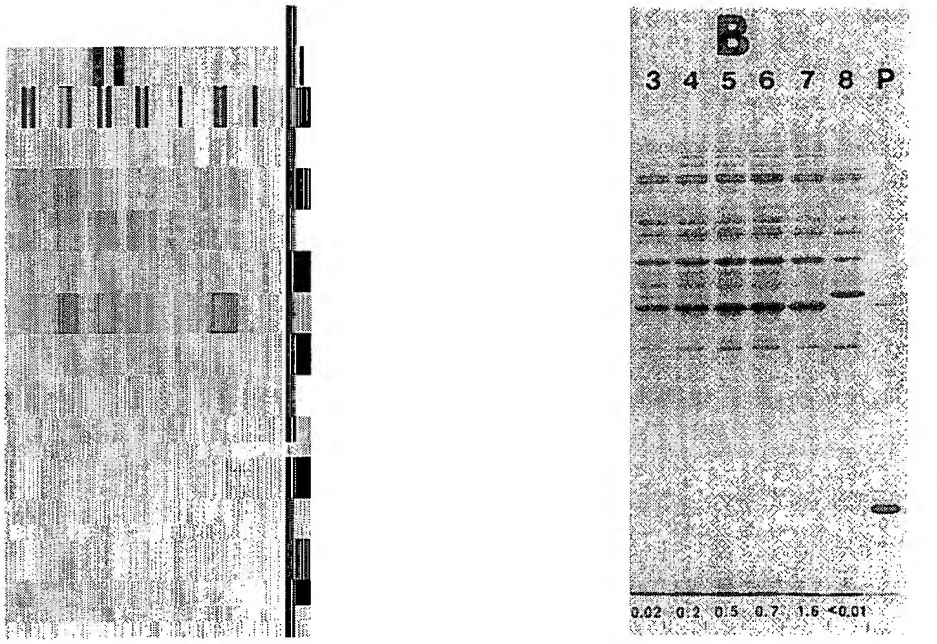

FIG. 3. Effect of amino acid supplementation on expression of OmpT, OmpU, TcpA, and cholera toxin (CT) in wild-type and *toxR55* mutant strains. *V. cholerae* strains were grown at 30°C in M9-glycerol minimal medium without additions (lanes 1) or with supplementation with the four amino acids asparagine, arginine, glutamate, and serine each at a concentration of 0.75 mM (lanes 2), 1.5 mM (lanes 3), 3.1 mM (lanes 4), 6.3 mM (lanes 5), 12.5 mM (lanes 6), or 25 mM (lanes 7 and 8). Whole-cell lysates were prepared in sample buffer and analyzed by SDS-PAGE. The amount of cholera toxin produced by a given culture is shown below its corresponding lane and is expressed as micrograms per optical density unit at 600 nm. (A) Results for strains O395 (lanes 1 to 7) and its *toxR55* mutant O395-55 (lane 8). (B) Results for strains CA401 (lanes 1 to 7) and its *toxR55* mutant CA401-55 (lane 8). A partially purified preparation of the TCP pilus was loaded in lanes P, and the positions of the OmpT, OmpU, and TcpA proteins are indicated by arrows, from top to bottom, respectively. Lane 5 of panel A was underloaded by one half.

with its replacement by two new fragments (fragments B and C for 569B-55). The same integration events occurred for strains O395-55 and CA401-55, but because these strains differ from strain 569B by a deletion mutation downstream from *toxR* (21), the hybridization pattern for these derivatives showed different but analogous bands in this analysis. It is also apparent from the diagram of the integration event that the chromosomal insertion of plasmid pVM55 creates two half copies of the *toxR* gene, but only the promoter-proximal half of the copy (Fig. 2B) carried sequences encoding the amino-terminal end of the ToxR protein. Thus, it can be predicted that this half copy (the *toxR55* allele) encodes a truncated ToxR peptide that lacks amino acid residues encoded by DNA downstream of the *Hpa*I site located at nucleotide 988 of the *toxR* gene (22), because this restriction enzyme site was used to construct pVM55 (Fig. 1). Similarly, *toxR* mutations constructed by integration of pJM703.12 would result in a half copy (the *toxR12* allele) encoding only the first 131 amino acid residues of ToxR (the *toxR* gene having been truncated at the *Nru*I site located at nucleotide 593) (22).

Characterization of *toxR* insertion mutants. Because the mutant phenotypes of derivatives of strains O395, CA401, and 569B carrying either the *toxR55* or *toxR12* alleles were similar, we focused primarily on characterizing the effect of the *toxR55* mutation. The *toxR55* mutants O395-55 and CA401-55 of strains O395 and CA401 were analyzed for total protein profile by SDS-PAGE and for toxin production by GM1 enzyme-linked immunosorbent assay when grown in M9 minimal medium supplemented with amino acids (Fig. 3). This medium allowed near-optimal expression of cholera toxin on a per cell basis for the parental strains. As indicated below lanes 7 and 8 in Fig. 3A and B, the *toxR55* mutant strains O395-55 and CA401-55 produced no detectable cholera toxin in this medium. Compared with their respective parental strains grown under identical conditions, these *toxR55* mutants produced at least 100- to 1,000-fold less toxin. Mutants with this toxin-deficient phenotype were similar to previously described mutagen-induced *toxR* mutants (6, 17). The total cellular protein profile of the *toxR55* mutants was altered relative to those of their parental strains, with the most prominent differences associated with the expression of three proteins called TcpA, OmpU, and OmpT (Fig. 4). We have previously shown (31) that the TcpA protein is the major subunit of the TCP pilus colonization factor and present evidence below that OmpU and OmpT are major outer membrane proteins of *V. cholerae*. We have also shown previously (31) that the changes in toxin, TcpA, OmpU, and OmpT expression seen in the *toxR55* mutants O395-55 and CA401-55 can be complemented by introduction of the ToxR$^+$ plasmid pVM53-D into these two strains, confirming that these pleiotropic protein expression effects are associated with the loss of *toxR* gene function. Thus, certain gene products like CtxA, CtxB, TcpA, and OmpU require *toxR* for expression, while other gene products like OmpT are apparently expressed maximally in the absence of a functional *toxR* gene.

Outer membrane protein expression in strain 569B. Strain 569B produced less OmpU and more OmpT than did strains O395 and CA401, so that the loss of OmpU expression in its

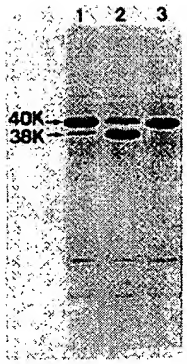

FIG. 4. Analysis of outer membrane preparations of strain 569B derivatives. *V. cholerae* strains were grown in CYE medium at 30°C, and outer membranes were prepared and analyzed on a SDS-12.5% polyacrylamide gel. The two major bands observed had apparent molecular weights of 40,000 (40K) and 38,000 (38K) and corresponded to OmpT and OmpU, respectively, in whole-cell lysates. Lanes: 1, 569B; 2, 569B(pVM7); 3, 569B-55.

toxR55 mutant (569B-55) was not as apparent in whole-cell lysates. Accordingly, we used outer membrane preparations to follow the response of OmpU and OmpT in this strain. Strain 569B produced two major outer membrane proteins of 40 and 38 kilodaltons (Fig. 5). These two proteins comigrated with the OmpT and OmpU proteins produced by *toxR55* mutants of O395 and CA401 and their respective parents (data not shown). Like with strains O395 and CA401, introduction of the *toxR55* allele into strain 569B resulted in the loss of the 38-kilodalton OmpU protein (Fig. 4, lane 3): this phenotype was complemented by the ToxR$^+$ plasmid pVM53-D (data not shown). Moreover, introduction of the high-copy-number ToxR$^+$ plasmid pVM7 (20) into the parental 569B strain resulted in increased OmpU expression and a reduction in OmpT expression (Fig. 4, lane 2). Introduction of pVM7 into an *N*-methyl-*N'*-nitrosoguanidine-induced *toxR* mutant derivative of 569B called M13 (6, 20) produced similar results (Fig. 5), indicating that the pVM7-encoded *toxR* gene is sufficient to cause this shift in expression of OmpU and OmpT.

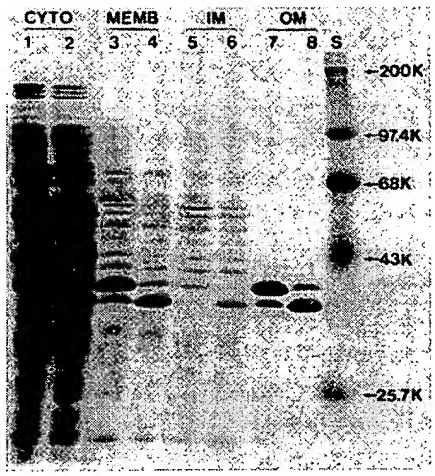

FIG. 5. Cell fractionation of M13(pBR322) and M13(pVM7). Strain M13 carrying either pBR322 or pVM7 was incubated at 30°C in CYE and then fractionated into cytoplasm (CYTO), total membrane (MEMB), Triton X-100-soluble (IM), and Triton X-100-insoluble (OM) fractions. Samples were analyzed by SDS-PAGE. Lanes 1, 3, 5, and 7 contained samples from M13(pBR322); lanes 2, 4, 6, and 8 contained samples from M13(pVM7). Lane S contained a sample of prestained protein standards with the indicated molecular weights: α-chymotrypsinogen, 25,700 (25.7 K); ovalbumin, 43,000 (43K); bovine serum albumin, 68,000 (68K); phosphorylase *b*, 97,400 (97.4K); myosin heavy chain, 200,000,000 (200K).

Fractionation of bacterial cells of strains M13(pBR322) and M13(pVM7) indicated that OmpT and OmpU represent the most abundant outer membrane proteins produced by these two derivatives of strain 569B (Fig. 5). Similar experiments with strains O395 and CA401 led to the same conclusion. Moreover, polyclonal antiserum raised against OmpU only weakly cross-reacts with OmpT, indicating that these are two distinct outer membrane proteins (V. DiRita and J. Mekalanos, unpublished data).

Physiological and nutritional parameters affecting expression of outer membrane proteins and ToxR-regulated genes. The observation that either mutations in *toxR* or the presence of multicopy ToxR$^+$ plasmids can produce changes in the expression of OmpT and OmpU suggests that the genes for these two proteins might be regulated by ToxR. Accordingly, we examined whether OmpT and OmpU expression is affected by some of the physiological parameters known to alter the expression of the ToxR-regulated *ctxAB* operon and *tcpA* gene in strain O395.

Strains O395 and CA401 produced, on a per cell basis, higher levels of cholera toxin and TcpA in glycerol-M9 minimal broth as the concentration of amino acids in this medium was increased from 0 to 25 mM (Fig. 3, lanes 1 to 7). In addition, growth was stimulated about twofold as the amino acid concentration was increased across this range. The amount of OmpU increased with increasing amino acid concentration in M9 minimal broth, showing that OmpU expression parallels that of other genes activated by *toxR*. In contrast, OmpT expression was optimal in the absence of amino acids and decreased as amino acids were added to this medium in higher concentrations. This negative response paralleled the increase in OmpT expression seen after inactivation of the *toxR* genes in these two strains by integration of pVM55 (Fig. 4, lane 8), suggesting that production of OmpT may be negatively controlled by *toxR* (31).

OmpT also showed an opposite response in its expression pattern relative to those of cholera toxin, TcpA, and OmpU in 1% tryptone broth containing various levels of NaCl (Fig. 6). In strain O395, concentrations of NaCl above 66 mM decreased the expression of cholera toxin, TcpA, and OmpU, while it increased the level of OmpT expression. However, this effect was biphasic, inasmuch as lower levels of NaCl (i.e., less than 50 mM) had an inhibitory effect on cholera toxin, TcpA, and OmpU production (data not shown). Growth yield was optimal at 132 mM NaCl and dropped off less than twofold above and below this level of salt.

To help quantify these effects, we used the recently described strain RT110.21, a derivative of strain O395 (31) which carries a *tcpA*::Tn*phoA* fusion, and measured the alkaline phosphatase (PhoA) activity produced by this strain under a variety of different growth conditions (Fig. 7). Strain O395 was used as a *tcpA*$^+$ control to assess the contribution of endogenous *V. cholerae* phosphatases to total PhoA activity observed in RT110.21.

From the results presented in Fig. 7, a number of conclusions can be drawn from comparison of the production of the TcpA protein (lanes 1), PhoA activity (lanes 2), and cholera

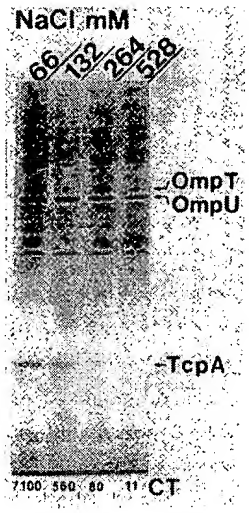

FIG. 6. Effect of NaCl concentration on expression of OmpT, OmpU, TcpA, and cholera toxin (CT) in tryptone broth. *V. cholerae* O395 was grown at 30°C in 1% tryptone broth (pH 6.5) containing the indicated amounts of NaCl. Whole-cell lysates were prepared in sample buffer and analyzed by SDS-PAGE. The amount of cholera toxin produced by each culture is indicated below each lane and is expressed as nanograms per optical density unit at 600 nm.

toxin (lanes 1 or 2). The requirement for the addition of 50 mM NaCl to tryptone broth to obtain high expression of these two *toxR*-regulated genes was satisfied by the addition of KCl at the same molarity. These two salts probably affect expression of *toxR*-regulated genes through their influence on the osmolarity rather than the ionic strength or salinity of the medium (8, 30), because lactose and melibiose (but not glycerol) largely substitute for salts when added to the same solute molarity. Lactose and melibiose are not metabolized by *V. cholerae* and therefore presumably act by increasing the osmolarity of the medium without influencing its ionic strength. Glycerol does not substitute for these osmoactive sugars or salts because it enters cells by facilitated diffusion. Its concentration thus equilibrates inside and outside the cell, producing no net change in the turgor pressure across the cell membrane (11).

The expression of OmpU and OmpT did not respond as dramatically to the absence of salt (Fig. 7) as it did to the presence of high levels of salt (Fig. 6) or to the supplementation of minimal medium with amino acids (Fig. 3). Moreover, at the optimal salt concentrations, variation of the incubation temperature (30 versus 37°C) and pH (6.5 versus 8.0) of the starting medium strongly influenced the expression of TcpA and cholera toxin in a coordinate fashion but had significantly less influence on the expression of OmpU and OmpT (Fig. 7). These data indicate that quantitative differences in expression can occur between certain *toxR*-regulated genes when their responses to various nutritional, physical, or environmental parameters are measured.

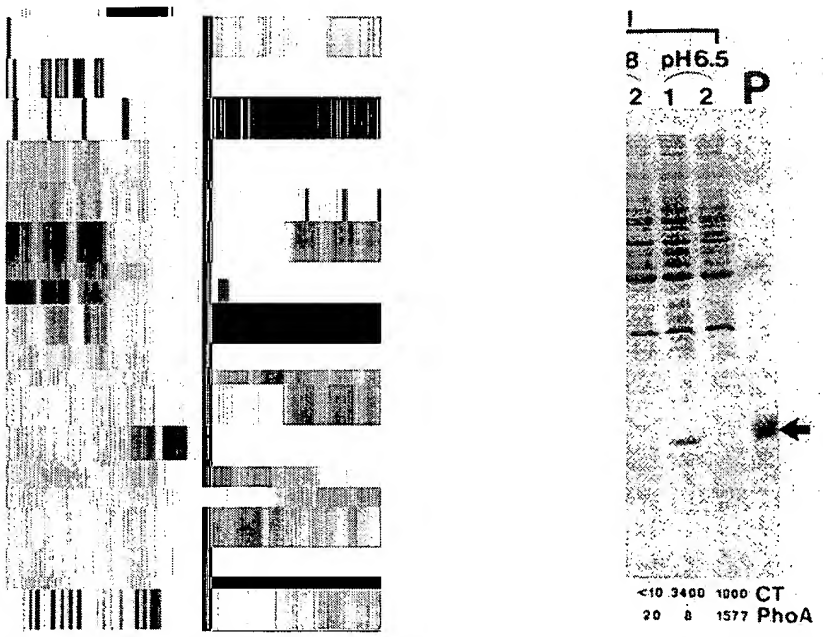

FIG. 7. Effect of osmoactive compounds, temperature, and pH on expression of OmpU, cholera toxin (CT), TcpA, and a TcpA-PhoA fusion protein. Two strains, O395 (lanes 1) and RT110.21 (lanes 2) were grown in 1% tryptone broth at 30°C (pH 6.5) with alterations in the medium composition, pH, or incubation temperature as indicated above the lanes. NaCl and KCl were added to 50 mM, and glycerol (GLY), lactose (LAC), and melibiose (MEL) were added to a 100 mM final concentration. Cultures in the lanes under the bracket on the right-hand side contained 0.5% yeast extract and 50 mM NaCl. The pH was adjusted with 4 N HCl or NaOH after the medium was autoclaved. The lane marked P contained a sample of partially purified TCP pilus. The arrows indicate the running position of the TcpA protein, and the star indicates the position of OmpU protein. Production of cholera toxin (CT) by each culture is indicated below the corresponding lanes and is expressed in nanograms per optical density unit at 600 nm. Alkaline phosphatase (PhoA) activities (units per optical density unit at 600 nm) due to the TcpA-PhoA fusion protein produced by strain RT110.21 are indicated below lanes 2. The PhoA activities shown below lanes 1 represent background due to *V. cholerae* phosphatases.

DISCUSSION

In this report we have described the construction and characterization of strains of *V. cholerae* carrying chromosomal insertion mutations in the *toxR* gene. These mutations were site directed by the use of specific internal fragments of the *toxR* gene to target the integration of a nonreplicating plasmid into the chromosomal *toxR* gene by homologous recombination (Fig. 2). Integration of pVM55 was expected to cause the truncation of the *toxR* gene at its *Hpa*I site (removing 31 codons from its 3′ end), while integration of pJM703.12 was expected to cause the truncation of *toxR* at its *Nru*I site (removing 88 codons from its 3′ end). Both types of mutations (*toxR55* and *toxR12*) produced similar phenotypes, as exemplified by strain O395-55, which produced no detectable TcpA protein and 1,000-fold less cholera toxin than did its parental strain. This is apparently the phenotype of a *toxR* null mutation because it is identical to the phenotype produced by an early deletion-frameshift mutation in *toxR* (the *toxR43* allele) carried by strain JJM43 (31).

In addition to their effects on toxin and pilus production, *toxR* null mutations produced changes in the expression of two major outer membrane proteins of *V. cholerae*. In strains O395, CA401, and 569B, the loss of a functional *toxR* gene resulted in a decrease in OmpU expression, with a concomitant increase in OmpT expression. In contrast to strains O395 and CA401, strain 569B produced comparably low levels of OmpU and high levels of OmpT before the inactivation of *toxR*. This ratio of expression was converted to the O395 and CA401 pattern by introduction of the high-copy-number *toxR*$^+$ plasmid pVM7 into 569B.

These genetic data suggest that ToxR regulates OmpU and OmpT expression and prompted us to do physiological studies on the expression of these outer membrane proteins and other *toxR*-regulated gene products under different growth conditions. We found that the addition of amino acids to glucose minimal broth or alteration of the osmolarity of tryptone broth produced coordinate changes in the expression of OmpT, OmpU, cholera toxin, and TcpA, with the expression of OmpT always following a pattern opposite those of OmpU, cholera toxin, and TcpA. In contrast, changes in either the starting pH of the growth medium or the incubation temperature had a marked effect on the expression of cholera toxin and TcpA but little or no effect on the expression of OmpT and OmpU. These data suggest that not all *toxR*-regulated genes respond to exactly the same nutritional and physical growth parameters. It is possible that these quantitative differences in expression of ToxR-regulated genes may reflect a fine-tuning of the regulatory response during the pathogenesis cycle. For example, OmpU might be most useful early in the cycle (e.g., contributing to survival in gastric juices or in the intestinal lumen), whereas cholera toxin and TCP are probably most important later, during the stage of mucosal surface colonization. Similarly, OmpT and proteins whose expression seems to be repressed by ToxR may be important to the survival of *V. cholerae* in the environment.

A variety of mechanisms other than direct transcriptional control by ToxR could explain the changes in outer membrane protein content of *toxR* mutants, as well as the differences in the degree of coordinate regulation of OmpT and OmpU relative to those of cholera toxin and TcpA. It is known, for example, that regulatory mechanisms exist in *E. coli* that bring about the induction of certain outer membrane proteins in response to the mutational loss of another outer membrane protein (4). The fluctuation in relative levels of OmpT and OmpU observed in this study was also reminiscent of the osmoregulation of *E. coli* porin proteins OmpC and OmpF, which is known to involve multiple regulatory mechanisms (4, 5). Thus, conclusive evidence that ToxR regulates the transcription of *ompU* and *ompT* will require the characterization of these genes and their promoters.

Results of this study have expanded earlier work by other investigators (3, 11, 24–26, 30) aimed at understanding the regulatory signals affecting expression of toxin production by *V. cholerae*. By following the expression of not only cholera toxin but also several other *toxR*-regulated gene products or PhoA fusion proteins, we were able to define certain physiological parameters that routinely affect expression in laboratory media of the *toxR* regulon of *V. cholerae* O395. In our study, like in previous ones, we did not conclusively identify the biologically relevant signals controlling the expression of *toxR*-regulated virulence factors in vivo (i.e., in the intestinal environment).

Temperature affects the expression of the *toxR* regulon, but the optimal temperature range for this parameter (22 to 30°C) is well below that found in animal tissues (37°C). Similarly, while *V. cholerae* is transiently exposed to the low pH of the stomach, it undergoes most of its growth in the upper intestine, where the pH is thought to be alkaline. Although low incubation temperature and low pH might produce maximum expression of some *toxR*-regulated genes, these culture conditions are not optimal for the growth of *V. cholerae* and do not significantly affect the expression of certain *toxR*-regulated gene products (e.g., OmpU). Accordingly, it is tempting to downplay the role of temperature and pH as important in vivo signals for the ToxR regulatory protein.

In contrast, the optimal osmolarity for cholera toxin, TCP, and OmpU production in tryptone broth is within a range that could conceivably represent that of mucosal secretions (e.g., serum has an osmolarity of about 300 mosM, which is equivalent to 150 mM NaCl) and that is also near the optimal osmolarity for growth of *V. cholerae*. Similarly, the presence of amino acids in minimal media had a stimulatory effect on both growth of *V. cholerae* and production of cholera toxin, TCP, and OmpU. There is little doubt that *V. cholerae* is exposed to amino acids that are released through the action of its own proteases and enzymes on the intestinal mucus.

Could osmolarity and the presence of amino acids be two important in vivo signals recognized by the ToxR regulatory protein? Are these two signals related to each other in some logical fashion? Perhaps. Osmotic stress is controlled by enteric bacteria, in part by alteration of intracellular pool sizes of certain amino acids or derivatives (e.g., proline and glutamate) through the induction of transport and biosynthetic pathways (12). In this regard, we have recently found that certain amino acids (e.g., glutamate or asparagine) which induce ToxR-regulated genes in minimal media can also act as osmoprotectants for *V. cholerae* in minimal media containing high inhibitory levels of NaCl (unpublished data). These results suggest that amino acids probably play a direct role in the osmoregulation in *V. cholerae*.

It has been proposed that osmoregulation in *E. coli* involves the ability of the bacterial cells to sense turgor pressure differences across their inner membrane (11, 12). Two regulatory proteins (EnvZ and KdpD) of *E. coli* have been hypothesized to be membrane proteins based on their postulated roles as osmosensors (5, 11, 12). We have previously reported (22) evidence that ToxR may be an osmosensor based on its transmembrane structure and its regulatory

properties. Interestingly, we also found (22) that a ToxR-PhoA fusion protein which lacks the C-terminal periplasmic domain of the ToxR polypeptide is no longer responsive to high osmolarity as a regulatory signal but is still responsive to the presence or absence of amino acids in the growth medium. While the interpretation of this mutant phenotype can vary, it does suggest that the amino acid sensory domain of ToxR is associated with its cytoplasmically located N terminus. The location of this domain is consistent with the notion that ToxR may be capable of sensing intracellular pool sizes of certain amino acids during the process of osmoregulation. A considerable amount of investigative work needs to be done to support the validity of this hypothesis. Nonetheless, it would be of interest to determine whether the expression of virulence determinants by other pathogenic bacteria responds to osmoregulatory signals and amino acids.

In this report we have also introduced the use of a novel vector for construction of chromosomal insertion mutations. The most useful properties of pJM703.1 include its ability to be mobilized at a high frequency by broad-host-range P-group conjugation functions and its transcomplementable R6K replication origin. The latter property makes pJM703.1 derivatives excellent suicide vectors for the introduction of DNA into bacterial cells in a nonreplicating form. Accordingly, derivatives of pJM703.1 have been shown to be useful broad-host-range delivery vectors for transposons such as Tn*phoA* (R. K. Taylor, C. Manoil, and J. J. Mekalanos, submitted for publication). Plasmid pGP704, a derivative of pJM703.1 containing a polylinker providing multiple cloning sites, facilitates marker exchange and mutant construction in *V. cholerae*, *Yersinia enterocolitica*, and *Bordetella pertussis* (G. Pearson, S. Knapp, V. DiRita, S. Bortner, R. Isberg, and J. J. Mekalanos, unpublished data). Thus, pJM703.1 can be used to introduce DNA constructions in a single copy into the chromosome of a wide range of bacterial species and therefore should be a valuable vector in genetic engineering.

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